AD_____

Award Number: W81XWH-11-1-0655

TITLE: Identification of Genetic Co-Modifiers in Shwachman-Diamond Syndrome

PRINCIPAL INVESTIGATOR: Sergei Revskoy, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University
Chicago, IL 60611-3008

REPORT DATE: August 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2012	Annual	30 July 2011 – 29 July 2012

4. TITLE AND SUBTITLE

Identification of Genetic Co-Modifiers in Shwachman-Diamond Syndrome

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0655

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Sergei Revskoy, Ph.D.

E-Mail: ferguson-m@northwestern.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Northwestern University
Chicago, IL 60611-3008

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of this proposal was to develop a powerful model for Shwachman-Diamond Syndrome that could ultimately be used to identify genetic co-factors for the development of myelodysplastic syndrome/acute myeloid leukemia (MDS/AML). We have hypothesized that SBDS damages hematopoietic stem cell and/or stroma, and that through the power of zebrafish modeling, we can identify the cellular and genetic lesions that promote MDS/AML. This model will allow us to 1) to carry out large scale screening for genetic co-modifiers that promote leukemogenesis followed by their validation, and 2) to carry out drug screening for compounds to modify SBDS-positive cells. We have generated transgenic zebrafish line harboring SBDS gene promoter fused with fluorescent protein Cherry and demonstrated that the gene is broadly expressed during embryonic development in various tissues, including hematopoietic and digestive systems while the expression is sparsely distributed (stem cells?) in juvenile and adult animals. We have also initiated a study on generation of ZFN-induced mutations in SBDS gene. Currently, we have been able demonstrate that sbds-specific ZFN does induced mutation in the targeted gene and that this mutation is lethal in early embryos which, however, can be rescued by SBDS-specific mRNA. This study creates a foundation for establishment of a unique animal model of SBDS.

15. SUBJECT TERMS
Zebrafish, animal model, leukemia, SBDS gene, reporter transgenes

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

INTRODUCTION:

The goal of this proposal is to develop a powerful model for Shwachman-Diamond Syndrome that could ultimately be used to identify genetic co-factors for the development of myelodysplastic syndrome/acute myeloid leukemia (MDS/AML).

Shwachman-Diamond Syndrome is an autosomal recessive bone marrow failure syndrome which carries a ~40% lifetime risk of developing acute myeloid leukemia or myelodysplastic syndrome (AML/MDS). The recently cloned Shwachman-Bodian-Diamond Syndrome (SBDS) gene has no homology with known genes.

Fundamental gaps in our knowledge about the function of SBDS exist. What cellular function does it serve? How does loss-of-function mutation in SBDS affect almost exclusively the hematopoietic, skeletal, and pancreatic tissues? What other factors are required for its transformation into MDS/AML? How can we use molecular, cellular, or genetic information to best improve the quality of life and prevent life-threatening complications for patients with Shwachman-Diamond Syndrome?

We have hypothesized that **loss of SBDS would damage the hematopoietic stem cell and/or stroma, which predisposes for the development of secondary MDS/AML, and that through the power of zebrafish modeling, we can identify the cellular components (stem cell v. stroma) and additional genetic lesions that promote leukemogenesis.**

To address this hypothesis, we proposed the following **specific aim**: establish an adult model of SDS in zebrafish using morpholino-induced gene knockdown. Having developed a zebrafish model for Shwachman-Diamond Syndrome, we and the scientific community can exploit it for 1) understanding developmental cues and processes involved in hematopoietic and pancreatic development, 2) establishing the roles of specific components of stromal cells, e.g. endothelial cells v. osteoblasts v. fibroblasts through transplantation and transgenic manipulation with specific genes, 4) large scale screening for genetic co-modifiers that promote leukemogenesis followed by their validation, 4) interrogation of human 7q candidate genes or GCSFR signaling molecules in the background of SBDSdeficiency, and 5) drug screening for compounds to restore normal function to SBDS-defective stem cells or stroma.

The gene responsible for SDS has been isolated (7q11.21), cloned, and characterized as the Shwachman-Bodian-Diamond Syndrome (SBDS) gene product; yet, little is known about its physiological function.4 The SBDS protein (250 amino acid, Mr 28,764 Da) has been linked to ribosome biogenesis, mitotic spindle stabilization, senescence, and chemotaxis.5 The syndrome

can be protean with variable skeletal abnormalities, pancreatic insufficient, and bone marrow failure.5 Neutropenia characterizes the primary defect in SDS, however, the degree of neutropenia can fluctuate and panctyopenia commonly occurs. Skeletal defects (e.g. metaphyseal dysplasia or polydactyly) are associated with lower numbers of osteoclasts and osteoblasts with reduced trabecular bone. These clinical findings support the use of SDS as a model to study perturbed blood stem cell-stromal cell interactions. A leading theory for the development of myelodysplasia is that the stroma instructs stem cells to undergo dysplasia and apoptosis via aberrantly produced cytokines. One major target for lenalidomide, approved for MDS, is the diseased stroma, although its precise mechanism of action(s) is poorly understood.8

When AML/MDS arises in patients with SDS, additional mutations occur: commonly cytogenetic abnormalities involving chromosome 7 (~67% of SDS patients)9 or a gain-in-function mutation of Ras.10 The gene(s) on chromosome 7 responsible for the disease progression are not known. A curious distinction in SDS is that some develop isochromosome i(7)(q10), but these patients do not develop MDS/AML. Indeed, this clinical finding demonstrates the importance of specific gene defects in driving disease and therapeutic interventions and underscores the importance of identifying more completely the genetic co-factors involved in leukemogenesis. We propose that investigation of a zebrafish model will provide information to questions too difficult to answer because of the rarity of the disease and the unpredictability in occurrence of MDS/AML.

Deficiency of SBDS damages the hematopoietic stem cell and/or stroma, which predisposes for the development of secondary MDS/AML, and that through the power of zebrafish modeling, we wish to identify the cellular components (stem cell v.stroma) additional genetic lesions that promote leukemogenesis. We will ultimately validate the model by creating an SBDSdeficient, KRasV12 transgenicstrain (beyond the budget and scope of this application). Then, we will identify the roles of stem cell and stroma by using our novel zebrafish transplantation protocol. First, we have developed develop the zebrafish model.

BODY:

Zebrafish as model for leukemogenesis. The morphology, molecular mechanisms of induction, and biological behavior of malignant tumors in zebrafish are very similar to those in mammals. In addition to similar mechanisms of tumor induction and gene expression

signatures, zebrafish tumors demonstrate the ability of invasive growth and metastasis and can be successfully transplanted to either syngeneic or immunosuppressed recipients. We have established the protocol for transplantation of zebrafish-derived leukemia.

We transplanted the leukemia line ZL1, induced by injection of *zRag2-EGFP-mMyc* fusion gene construct, described elsewhere14, into inbred CG2 clonal zebrafish embryos at 1-cell stage. The tumor growth was first detected in a 3-week old fish as a typical green fluorescence around the thymus area. Currently, the ZL1 leukemia line has already undergone more than 20 consecutive engraftments in syngeneic animals. During this period leukemia did not reveal substantial changes in cell morphology, biological behavior and the levels of GFP expression.

Progressive tumor growth in adult animals occurs mostly in the peritoneal cavity ultimately leading to almost synchronous host lethality within 9–12 days after an intraperitoneal engraftment of the leukemia cells. Grossly, the tumor emerged as a moderate enlargement of abdominal area, which reaches its maximum 2–3 days prior fish death. At necropsy, the tumor appears as a white jelly mass filling the entire peritoneal cavity of the fish. This mass demonstrates homogenous GFP fluorescence under a fluorescent stereomicroscope (**Fig. 1**). The leukemia cells can easily be dissociated by gentle pipetting the tumor in phosphate buffered saline and show minimal contamination with GFP negative cells, mostly erythrocytes. Injection of 4.6 nl of the leukemic cell suspension (1.0 or 2.5 x 10^7 cells/ml) into the peritoneal cavity of 5-day old larvae leads to emerging signs of leukemia such as intensive GFP fluorescence around thymus areas and peritoneum followed by enlargement of the abdominal area as soon as 4–5 days after the engraftment (**Fig. 2D–F**). The growth rate of transplanted leukemia directly correlates with the quantity of engrafted cells. Beginning day 7, the leukemia-bearing larvae are gradually becoming less mobile, are settling at the bottom of the well and stopped eating, i.e. demonstrate reliable signs of imminent death within 24 hours. The experiments utilizing leukemia-bearing larvae can be carried out in a multiwell plate format, which yields great potential for high throughput assays. In addition, this model has been proven efficient in quantitative assays for leukemia-initiating cells.14 We have also generated a variety of zebrafish models to further facilitate in-depth analysis of invasive tumor growth, angiogenesis, metastasis and tumor-initiating cells by in vivo imaging and provide a cost-effective system for high-throughput (HTP) screening of anticancer therapeutics, including biological response modifiers. The whole procedure, from generation of a gynogenetic female homozygous fish (a founder) to obtaining 3-4 consecutive passages of a syngeneic tumor, takes approximately 12-18 months. This time-frame largely depends on methods of tumor induction, tumor type and tumor growth

rate.15 More recently, we have demonstrated the efficacy of chemotherapeutic drugs broadly used for treatment of hematologic malignancies and soft tissue tumors in humans in an *in vivo* syngeneic model of transplantable acute lymphoblastic leukemia and rhabdomyosarcoma in zebrafish.16

Zebrafish model of SDS. Analysis of the zebrafish genome revealed the presence of the *SBDS* gene, encoding a protein with 90% homology to the human one. Unlike with the human gene, there is no adjacent pseudo-gene. Primers were designed and RNA harvested from developing zebrafish. RT-PCR demonstrated the presence of *SBDS* from after fertilization (**Fig. 3A).** Interestingly, qPCR showed that transcript decreased during embryogenesis (**Fig. 3B**). Little is known about the spatial and temporal expression of SBDS gene in adult tissues, our development of the SBDS promoter:eGFP will provide us a tool to advance our knowledge of SBDS in the mature animal).

Developmental expression patterns have not been reported for mouse or human, but the importance of *SBDS* is suggested by early embryonic lethality (ED 6.5) when deleted in the mouse.17 Due to high conservation at the amino acid level, zebrafish SBDS could be detected in western blotting (**Fig. 3C**). An initial set of experiments was performed using translational blocking morpholinos. Non-viability with developmental defects was observed in some, but not all injected embryos.

Decreased death rates with co-injection of SBDS RNA indicate that wastage was due to deficiency of the gene and not injection itself (**Fig 4**). Abnormal granulocyte distribution has been recently reported, suggesting that defects in myeloid development in the SBDS-deficient zebrafish.18

KEY RESEARCH ACCOMPLISHMENTS:

- Establishment of a leukemia model in zebrafish (Figure 1).
- Demonstration of similarity of early embryonic phenotypes induced by disruption of SBDS gene in mammals and zebrafish using gene knockout and gene knockdown by morpholinos, respectively.
- Demonstration of differences in regulation of SBDS promoter activity in mammals and zebrafish (Figure 2).
- Generation of tools and reagents, including antibodies, morpholinos and transgenic constructs, for further characterization of SBDS gene expression and function in zebrafish.

CONCLUSION:

We have demonstrated that SBDS gene is critical for early embryonic development in fish which recapitulates finding on its function in mammals. Our results demonstrate streaking similarities of the zebrafish models of SBDS and those in mammals. Now, different models of SBDS in zebrafish are available

Further studies such as syngeneic transplantation followed by tracing of SBDS-positive cells in juvenile and adult organism will provide direct evidence on the indispensible role of SBDS-positive cells in hematopoietic linage commitment and bone marrow failure. More importantly, these studies may help develop new strategies for the treatment of bone marrow failure, SDS in particular.

Literature Cited

1. Corey SJ, Minden MD, Barber DL, Kantarjian H, Wang JC, Schimmer AD. Myelodysplastic syndromes: the complexity of stem-cell diseases. Nat Rev Cancer. 2007;7:118-129.

2. Donadieu J, Leblanc T, Bader Meunier B, et al. Analysis of risk factors for myelodysplasias, leukemias and death from infection among patients with congenital neutropenia. Experience of the French Severe Chronic Neutropenia Study Group. Haematologica. 2005;90:45-53.

3. Rosenberg PS, Alter BP, Bolyard AA, et al. The incidence of leukemia and mortality from sepsis in patients with severe congenital neutropenia receiving long-term G-CSF therapy. Blood. 2006;107:4628-4635.

4. Boocock GR, Morrison JA, Popovic M, et al. Mutations in SBDS are associated with Shwachman-Diamond syndrome. Nat Genet. 2003;33:97-101.

5. Burroughs L, Woolfrey A, Shimamura A. Shwachman-Diamond syndrome: a review of the clinical presentation, molecular pathogenesis, diagnosis, and treatment. Hematol Oncol Clin North Am. 2009;23:233-248.

6. Toiviainen-Salo S, Mayranpaa MK, Durie PR, et al. Shwachman-Diamond syndrome is associated with low-turnover osteoporosis. Bone. 2007;41:965-972.

7. Rosenfeld C, List A. A hypothesis for the pathogenesis of myelodysplastic syndromes: implications for new therapies. Leukemia. 2000;14:2-8.

8. Verma A, List AF. Cytokine targets in the treatment of myelodysplastic syndromes. Curr Hematol Rep. 2005;4:429-435.

9. Dror Y. Shwachman-Diamond syndrome. Pediatr Blood Cancer. 2005;45:892-901.

10. Shimamura A. Shwachman-Diamond syndrome. Semin Hematol. 2006;43:178-188.

11. Minelli A, Maserati E, Nicolis E, et al. The isochromosome i(7)(q10) carrying c.258+2t>c mutation of the SBDS gene does not promote development of myeloid malignancies in patients with Shwachman syndrome. Leukemia. 2009;23:708-711.

12. Fleitz J, Rumelhart S, Goldman F, et al. Successful allogeneic hematopoietic stem cell transplantation (HSCT) for Shwachman-Diamond syndrome. Bone Marrow Transplant. 2002;29:75-79.

13. Smith AC, Raimondi AR, Salthouse CD, et al. High-throughput cell transplantation establishes that tumor-initiating cells are abundant in zebrafish T-cell acute lymphoblastic leukemia. Blood. 2010.

14. Langenau DM, Zon LI. The zebrafish: a new model of T-cell and thymic development. Nat Rev Immunol. 2005;5:307-317.

15. Mizgirev I, Revskoy S. Generation of clonal zebrafish lines and transplantable hepatic tumors. Nat Protoc. 2010;5:383-394.

16. Mizgirev IV, Revskoy S. A new zebrafish model for experimental leukemia therapy. Cancer Biol Ther. 2010;9.

17. Zhang S, Shi M, Hui CC, Rommens JM. Loss of the mouse ortholog of the shwachman-diamond syndrome gene (Sbds) results in early embryonic lethality. Mol Cell Biol. 2006;26:6656-6663.

SUPPORTING DATA:

Figures and tables

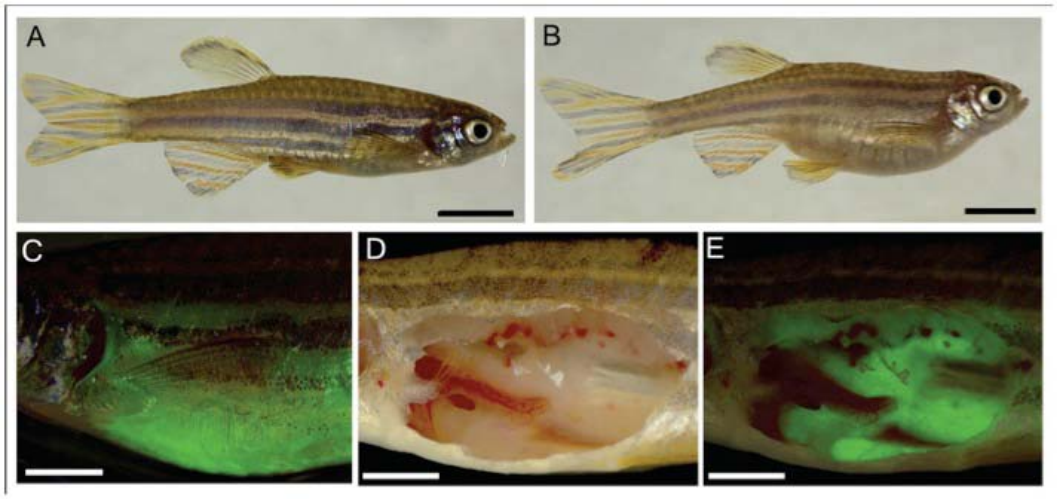


Figure 1. Leukemia growth in adult fish. Control (A) and lymphoblastic leukemia-bearing (B) fish 8 days after transplantation of leukemia cells. Grossly the tumor emerged as a moderate uniform enlargement of the abdominal area. GFP-fluorescence of leukemic cells in the peritoneal cavity was captured through body wall (C). Bright field image of the surgically opened abdomen (D) and digitally merged bright field and fluorescent images (E). The gelatinous mass mass represents leukemic tumor (D). Bar length is 5 mm for images (A and B) and 2.5 mm for (B–E).

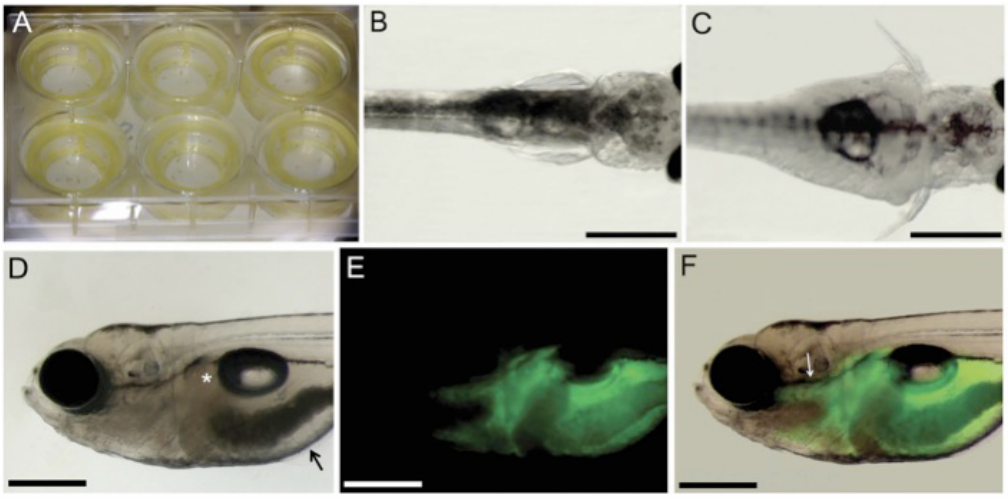
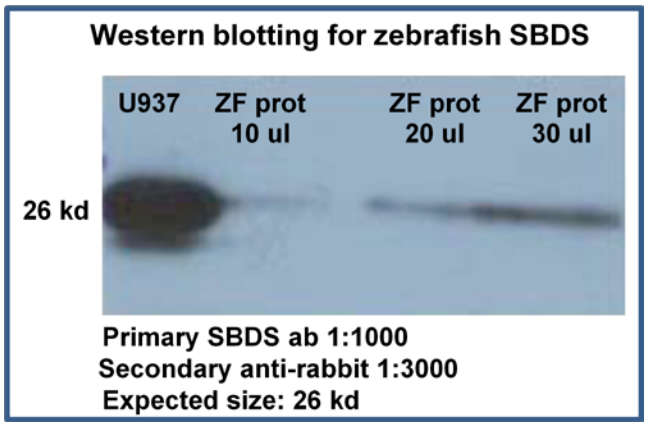


Figure 2. Leukemia growth in larvae. A6-well plate with inserted 100 µm Nylon Cell Strainer was able to house 10 larvae/well for up to 20 days (A). Dorsal view of 15 dpf control (B) and leukemia-bearing larvae (C) 10 days after leukemia cell engraftment. Typical enlargement of the larval body as a result of leukemia growth. Lateral view of 15 dpf larvae with massive infiltration of abdominal area spreading towards the thymus area 10 days after leukemia engraftment. Development of ascites (arrow) in peritoneal cavity of leukemia engrafted larvae is a typical symptom of terminal stage of leukemia progression; asterisk indicates a site of leukemia cell injection, bright field (D); wide field fluorescent microscopy (E) and digitally merged bright field and fluorescent images (F), leukemia cell homing to thymus area (arrow).

Figure 3.



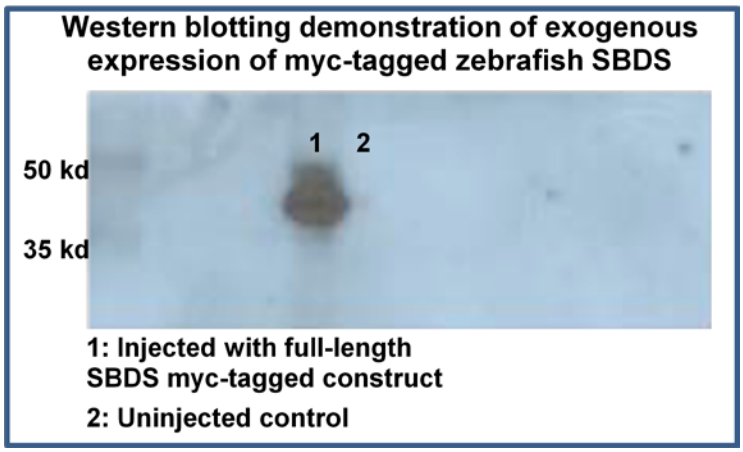
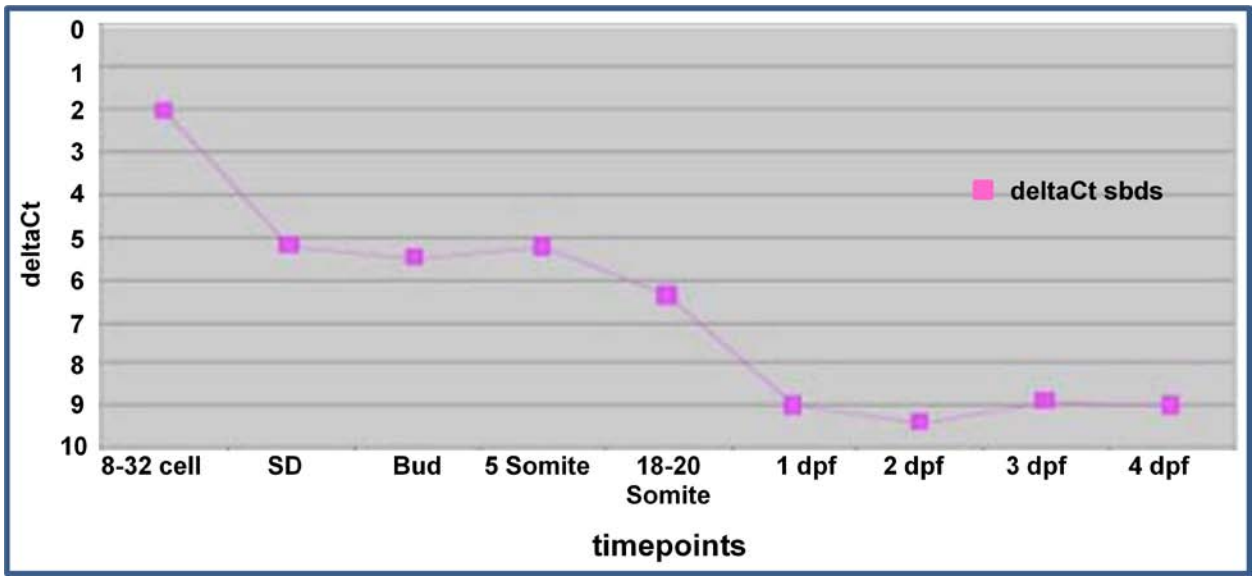


Figure 4.


0
1
2
3
4
5
6
7
8
9
10
deltaCt
deltaCt sbds
8-32 cell
SD
Bud
5 Somite
18-20 Somite
1 dpf
2 dpf
3 dpf
4 dpf
timepoints